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Title: EXPRESSION OF SOMATOTROPIN IN PLANT SEEDS

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CROSS REFERENCE TO RELATED APPLICATIONS

The present application is a continuation-in-part of USSN 08/846,021 that was filed on April 25, 1997 which is a continuation-in-part of USSN 08/366,783 that was filed on December 30, 1994 which is a continuation-in-part of USSN 08/142,418 that was filed November 16, 1993 which is a continuation-in-part of USSN 07/659,835 that was filed on February 22, 1991, all of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention provides novel transgenic plant seeds comprising a somatotropin as well as methods of preparing plant seeds comprising somatotropins.

BACKGROUND OF THE INVENTION

Naturally occurring somatotropins are polypeptides, the amino acid sequences of which for a number of vertebrate species have been reported. These include bovine (Miller et al., 1980, J. Biol. Chem, 255, 7251) porcine (Seeburg et al., 1983, DNA 2: 37), human (US Patent No. 3,853,832; Martial et al., Science, 205: 602-617) and various piscine somatotropins (e.g.: Sekine et al., 1985. Proc. Natl. Acad. Sci. (USA), 82: 4306-4310; Agellon et al., 1988, Proc. Natl. Acad. Sci. (USA), 85: 5136-5140; US Patent Nos. 4,689,402 and 4,894,362). In general, somatotropins isolated from different species display a high degree of amino acid sequence identity (Chang et al., 1992, Gen. and Comp. Endocrin. 87: 385-393). Analogs of somatotropins are also known. European Patent Application 103 395, for example discloses bovine somatotropin analogs. These analogs typically relate to the insertion, addition or deletion of nucleotides of the somatotropin gene thereby creating a protein different from the naturally occurring somatotropin.

The preparation of somatotropins is well known in the art. Bovine somatotropin, for example can be prepared by extraction from the pituitary tissue, (Li et al., 1954, J. Biol. Chem. 211: 55 and US Patent No. 4,371,462). Somatotropins can also be prepared by production in genetically engineered microorganisms, such as *Escherichia coli* containing recombinant DNA which encodes a somatotropin polypeptide (e.g. Seeburg et al., 1978,

Nature). US Patent No. 4,443,549 discloses a method for producing bovine somatotropin in yeast cells. Methods for high yield bovine somatotropin production in microorganisms are disclosed in US Patent Nos. 5,240,837 and 5,489,529.

Similarly, the preparation of human somatotropin is known. For example US Patent No. 5,637,495 and Mukhija et al. (Gene 165: 303-306) teach the production of human growth hormone in *E. coli*.

Fish somatotropins have also been produced in a variety of microorganisms. US Patent No. 5,270,180 for example discloses a method for the production of salmon growth hormone in *E. coli* and yeast and in US Patent No. 4,894,362 a microbial production system for eel growth hormone is disclosed.

The low costs associated with growing plants, make plants an attractive host for the production of somatotropins. To the best of the present inventors knowledge only one attempt has been reported to produce a somatotropin in plants. Bosch et al. (Transgenic Research, 1994, 3: 304-310) expressed a trout growth hormone in the leaves of transgenic tobacco plants, however they were unsuccessful in accumulating somatotropin in seeds.

Although methods for producing somatotropins are well known to skilled artisans, the existing methods are relatively expensive, especially when large production volumes are required. Accordingly there is a need in the art for additional economical production methods of somatotropin.

SUMMARY OF THE INVENTION

The present inventors have discovered a cost effective method for the preparation of somatotropins in the seeds of plants. The method involves expressing a somatotropin in plant seeds as an oleosin fusion protein so that the somatotropin has biological activity.

Accordingly, the present invention provides a method for the expression of a somatotropin in a plant comprising:

(a) introducing into a plant cell a chimeric nucleic acid sequence comprising:

(1) a first nucleic acid sequence capable of regulating the transcription in said host cell of

(2) a second nucleic acid sequence, wherein said second sequence encodes a recombinant fusion polypeptide and comprises (i) a nucleic acid sequence encoding a sufficient portion of an oleosin protein to provide targeting of the recombinant fusion polypeptide to a lipid phase, linked in frame to (ii) a nucleic acid sequence encoding said somatotropin; and

(3) a third nucleic acid sequence encoding a termination region functional in said plant cell; and

(b) growing said plant cell to produce said recombinant fusion polypeptide.

In a preferred embodiment of the invention, the somatotropin is fish growth hormone. In a further preferred embodiment of the invention the somatotropin is carp growth hormone.

In another aspect the invention provides a chimeric nucleic acid sequence, capable of being expressed in association with an oil body of a plant cell, comprising:

(1) a first nucleic acid sequence capable of regulating the transcription in said plant cell

(2) a second nucleic acid sequence, wherein said second sequence encodes a recombinant fusion polypeptide and comprises (i) a nucleic acid sequence encoding a sufficient portion of an oleosin protein to provide targeting of the recombinant fusion polypeptide to a lipid phase, linked in reading frame to (ii) a nucleic acid sequence encoding a somatotropin; and

(3) a third nucleic acid sequence encoding a termination region functional in said host cell.

In a further aspect, the instant invention provides plant seeds expressing a somatotropin. In one embodiment of the invention the somatotropin is a fish growth hormone.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 is a schematic diagram of the oleosin-cGH fusion construct.

Figure 2 shows The expression of oleosin-carp growth hormone fusion in protein in three generations of canola seed.

Figure 3 is the nucleic acid sequence (SEQ.ID.NO.:1) and deduced amino acid sequence (SEQ.ID.NO.:2) of the oleosin-cGH fusion sequence.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the production of somatotropins. Somatotropins, frequently referred to in the art as growth hormones, are anabolic hormones produced by the vertebrate pituitary gland. In general, somatotropins display pleiotropic biological effects, which include promotion of skeletal growth and stimulation of mammalian milk secretion. Accordingly it has been recognized that the use of somatotropins results in improvements of the production economics in industries such as aquaculture and the livestock industry.

As hereinbefore mentioned, the present invention provides transgenic plant seeds comprising a somatotropin as well as methods of preparing plant seeds comprising somatotropins.

Accordingly the present invention provides a method for the expression of a somatotropin in a plant cell comprising:

(a) introducing into a plant cell a chimeric nucleic acid sequence comprising:

(1) a first nucleic acid sequence capable of regulating the transcription in said plant cell of

(2) a second nucleic acid sequence, wherein said second nucleic acid sequence encodes a recombinant fusion polypeptide and comprises (i) a nucleic sequence encoding a sufficient portion of an oleosin protein to provide targeting of the recombinant fusion polypeptide to a lipid phase, linked in frame to (ii) a nucleic sequence encoding a somatotropin; and

(3) a third nucleic acid sequence encoding a termination region functional in said plant cell; and

(b) growing said plant cell to produce said recombinant fusion polypeptide.

The term "somatotropin" as used herein comprises any active somatotropin, including bovine, ovine, avian, canine, piscine, porcine, rat and human somatotropin or any biologically active analogs or fragments thereof, including somatotropin derivatives which have been obtained by, adding, deleting or substituting amino acids or by otherwise modifying the structure of any naturally occurring somatotropin. Throughout this application the term "somatotropin" is used interchangeably with the term "growth hormone".

The term "nucleic acid sequence" refers to a sequence of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The term also includes modified or substituted sequence comprising non-naturally occurring monomers or portions thereof, which function similarly. The nucleic acid sequences of the present invention may be ribonucleic (RNA) or deoxyribonucleic acids (DNA) and may contain naturally occurring bases adenine, guanine, cytosine, thymidine and uracil. The sequences may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2 propyl and other alkyl adenines, 5 halo uracil, 5 halo cytosine, 6-aza uracil, 6-aza cytosine, abd 6-aza thymine, pseudo uracil, 4-thiouruacil, 8-halo adenine, 8-amino adenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl, adenine and other 8-substituted adenines, 8-halo guanines, 8 amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-thrifluoromethyl uracil and 5-trifluoro cytosine.

The term " sufficient portion of an oleosin protein to provide targeting of the recombinant fusion polypeptide to a lipid phase" means any oleosin protein or any analog or portion thereof, including oleosin derivatives which have been obtained by, adding, deleting or substituting amino acids or by otherwise modifying the structure of any naturally occurring oleosin which is capable of targeting to a lipid phase. Lipid phase

is intended to mean any subcellular structure comprising triacylglycerides, including oil bodies and other organelles comprising membranes or membrane like structures such as the endoplasmatic reticulum or the chloroplast.

The nucleic acid and amino acid sequences of numerous somatotropins including, bovine (Miller et al., 1980, J. Biol. Chem, 255, 7251) porcine (Seeburg et al., 1983, DNA 2: 37), human (US Patent No. 3,853,832; Martial et al., Science, 205: 602-617) and various piscine somatotropins (e.g.: Sekine et al., 1985. Proc. Natl. Acad. Sci. (USA), 82: 4306-4310; Agellon et al., 1988, Proc. Natl. Acad. Sci. (USA), 85: 5136-5140; US Patent Nos. 4,689,402 and 4,894,362) are available. Analogs of somatotropins are also known (e.g. European Patent Application 103 395). In preferred embodiments of the present invention the somatotropin is a fish somatotropin. In a particularly preferred embodiment of the invention the somatotropin is a carp somatotropin. Based on the sequences cDNA clones comprising the genetic material encoding the somatotropins may be prepared and oleosin fusion genes may be prepared in accordance with the present invention and practicing techniques commonly known to those skilled in the art (see e.g. Sambrook et al. (1990), Molecular Cloning, 2nd ed., Cold Spring Harbor Press).

To identify other somatotropins having desired characteristics, a nucleic acid probe may be designed and prepared to identify additional somatotropins. The nucleic acid probe may be used to screen cDNA or genomic libraries from any living cell or virus. Sequences which hybridize with the probe under stringent conditions may then be isolated. Given the sequence identity of the somatotropins isolated from different species to date (Chang et al., 1992, Gen. and Comp. Endocrin. 87: 385-393) somatotropins from a broad range of species may be isolated according to this method.

Somatotropin sequences may also be isolated by screening expression libraries. Antibodies against existing somatotropins may be obtained and expression libraries may be screened with these antibodies essentially as described by Huynh et al. (1985, in DNA cloning, Vol 1, a

Practical Approach ed. D. M. Glover, IRL Press). Expression libraries may be prepared from any living cell or virus.

Other somatotropins may be discovered by those skilled in the art. The actual somatotropin sequence which is selected is not of critical importance and may be as desired. It is to be clearly understood that any somatotropin may be employed without departing from the spirit or scope of the present invention.

The chimeric nucleic acid sequences which encode the oleosin-somatotropin fusion proteins of the present invention can be incorporated in a known manner into a recombinant expression system which ensures expression in the plant host cell. Accordingly, the present invention also includes a recombinant expression vector comprising a chimeric nucleic acid sequence operatively linked to a regulatory sequence and termination region suitable for expression in a host cell. In one embodiment the invention provides a chimeric nucleic acid sequence, capable of being expressed in association with an oil body of a plant cell, comprising:

(1) a first nucleic acid sequence capable of regulating the transcription in said plant cell

(2) a second nucleic acid sequence, wherein said second sequence encodes a recombinant fusion polypeptide and comprises (i) a nucleic acid sequence encoding a sufficient portion of an oleosin protein to provide targeting of the recombinant fusion polypeptide to a lipid phase, linked in reading frame to (ii) a nucleic acid sequence encoding a somatotropin; and

(3) a third nucleic acid sequence encoding a termination region functional in said host cell.

The nucleic acid sequence encoding the somatotropin may be genetically fused upstream or downstream of the nucleic acid sequence encoding the oleosin protein and concatamers containing repetitive units of the somatotropin may be employed. In preferred embodiments, the somatotropin gene is fused downstream of the oleosin gene.

The present invention provides plant seeds which recombinantly express somatotropins. In a preferred embodiment of the

present these seeds are obtained from a dicotelydenous plant. In a yet further preferred embodiment the seeds are exalbuminous seeds. In a further preferred embodiment of the instant invention the plant seeds are obtained from the group of plant species comprising: rapeseed (*Brassica* spp.), linseed/flax (*Linum usitatissimum*), safflower (*Carthamus tinctorius*), sunflower (*Helianthus annuus*), maize (*Zea mays*), soybean (*Glycine max*), mustard (*Brassica* spp. and *Sinapis alba*), crambe, (*Crambe abyssinica*), eruca (*Eruca sativa*), oil palm (*Elaeis guineeis*), cottonseed (*Gossypium* spp.), groundnut (*Arachis hypogaea*), coconut (*Cocus nucifera*), castor bean (*Ricinus communis*), coriander (*Coriandrum sativum*), squash, (*Cucurbita maxima*), Brazil nut (*Bertholletia excelsa*) and jojoba (*Simmondsia chinensis*). It is expected that the somatotropin is expressed in all embryonic tissue, although difference in expression levels may be detected in different tissues of the embryonic axis and the cotyledon.

(I) <u>CLONING, PLANT TRANSFORMATION AND REGENERATION</u>

Cloning and Transformation Vectors

Two types of vectors are routinely employed. The first type of vector is used for the genetic-engineering and assembly of constructs and typically consists of a backbone such as found in the pUC family of vectors, enabling replication in easily-manipulated and maintained gram negative bacteria such as *E. coli*. The second type of vector typified by the Ti and Ri plasmids, specify DNA transfer functions and are used when it is desired that the constructs be introduced into the plant and stably integrated into its genome via *Agrobacterium*-mediated transformation.

A typical construct consists, in the 5′ to 3′ direction, of a regulatory region complete with a promoter capable of directing expression in plants (preferably seed-specific expression), a protein coding region, and a sequence containing a transcriptional termination signal functional in plants. The sequences comprising the construct may be either natural or synthetic or any combination thereof.

Both non-seed specific promoters, such as the 35-S CaMV promoter (Rothstein et al., 1987; Gene 53: 153-161) and seed-specific promoters such as the phaseolin promoter (Sengupta-Gopalan et al., 1985; PNAS USA 82: 3320-3324) or the Arabidopsis 18 kDa oleosin (Van Rooijen et

al., 1992; Plant Mol. Biol. 18: 1177-1179) promoters may be used. In addition to the promoter, the regulatory region contains a ribosome binding site enabling translation of the transcripts in plants and may also contain one or more enhancer sequences, such as the AMV leader (Jobling and Gehrke 1987; Nature 325: 622-625), to increase the expression of product.

The coding region of the construct will typically be comprised of sequences encoding a ligand fused in frame to an oleosin and ending with a translational termination codon. The sequence for the oleosin may be comprised of any DNA sequence, or part thereof, natural or synthetic, sufficient to encode a protein that can be correctly targeted to, and stably expressed on, an oil body. A detailed description of the characteristics of such a sequence has been reported previously in Moloney, 1993; PCT Patent Appl. WO 93/21320 which is hereby incorporated by reference. The sequence may also include introns. The ligand-encoding region may in turn be comprised of any individual, or combination of, ligand sequences identified as described above. If desired, a protease or chemical recognition site may be engineered between the ligand and the target protein to enable proteolytic removal of the ligand from the target protein in the course of purification.

The region containing the transcriptional termination signal may comprise any such sequence functional in plants such as the nopaline synthase termination sequence and additionally may include enhancer sequences to increase the expression of product.

The various components of the construct are ligated together using conventional methods, typically into a pUC-based vector. This construct may then be introduced into an *Agrobacterium* vector and subsequently into host plants, using one of the transformation procedures outlined below.

Transformation of Plants

A variety of techniques is available for the introduction of DNA into host cells. For example, the chimeric DNA constructs may be introduced into host cells obtained from dicotyledonous plants, such as tobacco, and oleaginous species, such as *B. napus* using standard *Agrobacterium* vectors; by a transformation protocol such as that described

by Moloney et al., 1989, (Plant Cell Rep., 8: 238-242) or Hinchee et al., 1988, (Bio/Technol., 6: 915-922); or other techniques known to those skilled in the art. For example, the use of T-DNA for transformation of plant cells has received extensive study and is amply described in EPA Serial No. 120,516; Hoekema *et al.*, 1985, (Chapter V, In: The Binary Plant Vector System Offset-drukkerij Kanters B.V., Alblasserdam); Knauf, et al., 1983, (Genetic Analysis of Host Range Expression by Agrobacterium, p. 245, In Molecular Genetics of the Bacteria-Plant Interaction, Puhler, A. ed., Springer-Verlag, NY); and An et al., 1985, (EMBO J., **4**: 277-284). Conveniently, explants may be cultivated with *A. tumefaciens* or *A. rhizogenes* to allow for transfer of the transcription construct to the plant cells. Following transformation using *Agrobacterium* the plant cells are dispersed in an appropriate medium for selection, subsequently callus, shoots and eventually plantlets are recovered. The *Agrobacterium* host will harbour a plasmid comprising the vir genes necessary for transfer of the T-DNA to the plant cells. For injection and electroporation, (see below) disarmed Ti-plasmids (lacking the tumour genes, particularly the T-DNA region) may be introduced into the plant cell.

The use of non-*Agrobacterium* techniques permits the use of the constructs described herein to obtain transformation and expression in a wide variety of monocotyledonous and dicotyledonous plants and other organisms. These techniques are especially useful for species that are intractable in an *Agrobacterium* transformation system. Other techniques for gene transfer include biolistics (Sanford, 1988, Trends in Biotech., 6: 299-302), electroporation (Fromm et al., 1985, Proc. Natl. Acad. Sci. USA, 82: 5824-5828; Riggs and Bates, 1986, Proc. Natl. Acad. Sci. USA 83: 5602-5606) or PEG-mediated DNA uptake (Potrykus et al., 1985, Mol. Gen. Genet., 199: 169-177).

In a specific application, such as to *B. napus*, the host cells targeted to receive recombinant DNA constructs typically will be derived from cotyledonary petioles as described by Moloney et al., (1989, Plant Cell Rep., 8: 238-242). Other examples using commercial oil seeds include cotyledon transformation in soybean explants (Hinchee et al., 1988. Bio/Technology, 6: 915-922) and stem transformation of cotton (Umbeck *et* al., 1981, Bio/Technology, 5: 263-266).

Regeneration and Analysis of Transgenic Plants

Following transformation, the cells, for example as leaf discs, are grown in selective medium. Once shoots begin to emerge, they are excised and placed onto rooting medium. After sufficient roots have formed, the plants are transferred to soil. Putative transformed plants are then tested for presence of a marker. Southern blotting is performed on genomic DNA using an appropriate probe, for example an *A. thaliana* oleosin gene, to show that integration of the desired sequences into the host cell genome has occurred.

The expression cassette will normally be joined to a marker for selection in plant cells. Conveniently, the marker may be resistance to a herbicide, e.g. phosphinothricin or glyphosate, or more particularly an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, chloramphenicol, or the like. The particular marker employed will be one which will allow for selection of transformed cells compared with cells lacking the introduced recombinant DNA.

The fusion peptide in the expression cassette constructed as described above, expresses at least preferentially in developing seeds. Accordingly, transformed plants grown in accordance with conventional ways, are allowed to set seed. See, for example, McCormick et al. (1986, Plant Cell Reports, 5: 81-84). Northern blotting can be carried out using an appropriate gene probe with RNA isolated from tissue in which transcription is expected to occur, such as a seed embryo. The size of the transcripts can then be compared with the predicted size for the fusion protein transcript.

Oil body proteins are then isolated from the seed and analyses performed to determine that the fusion peptide has been expressed. Analyses can be for example by SDS-PAGE. The fusion peptide can be detected using an antibody to the oleosin portion of the fusion peptide. The size of the fusion peptide obtained can then be compared with predicted size of the fusion protein.

Two or more generations of transgenic plants may be grown and either crossed or selfed to allow identification of plants and strains with desired phenotypic characteristics including production of recombinant

proteins. It may be desirable to ensure homozygosity of the plants, strains or lines producing recombinant proteins to assure continued inheritance of the recombinant trait. Methods of selecting homozygous plants are well know to those skilled in the art of plant breeding and include recurrent selfing and selection and anther and microspore culture. Homozygous plants may also be obtained by transformation of haploid cells or tissues followed by regeneration of haploid plantlets subsequently converted to diploid plants by any number of known means, (e.g.: treatment with colchicine or other microtubule disrupting agents).

(II) <u>RECOVERY OF THE POLYPEPTIDE</u>

The present invention also includes a fusion polypeptides encoded for by a chimeric nucleic acid sequence comprising (i) a nucleic acid sequence encoding a sufficient portion of an oil body protein to provide targeting of the fusion polypeptide to an oil body linked in reading frame to (ii) a nucleic acid sequence encoding a somatotropin. Preferably the fusion polypeptide displays growth hormone activity. Consequently, the present invention includes a fusion polypeptide comprising a somatotropin wherein the expressed somatotropin moiety is biologically active. In preferred embodiments of the invention, the fusion polypeptide comprises an oleosin protein fused to a somatotropin, wherein the chimeric protein displays growth hormone activity.

The invention further provides methods for the separation of the fusion protein from host cell components by partitioning of the oil body fraction. Optionally, the recombinant somatotropin may be released from the fusion protein via specific cleavage of the somatotropin - oil body protein fusion. Optionally a cleavage site may be located prior to the N-terminus and after the C-terminus of the somatotropin allowing the fusion polypeptide to be cleaved and separated by phase separation into its component peptides.

In preferred embodiments of the present invention, seeds are crushed upon harvesting by grinding, pulverizing or otherwise breaking open the seed cells using milling equipment, for example flaking rolls, disk mills, colloid mils, pin mills, orbital mills IKA mills or industrial style homogenizers. In one embodiment of the present invention, the crushed

seed fraction may directly be employed as an ingredient to formulate compositions, such as animal feed compositions, comprising somatotropin. In alternative embodiments of the invention a seed fraction comprising somatotropin is isolated. In a preferred embodiment of the present invention, the isolated seed fraction comprises intact oil bodies. In order to isolate the oil body fraction of the seeds, plant seeds are preferably first crushed. In one embodiment the crushed seed fraction is subsequently submitted to density centrifugation resulting in a separation of the oil body fraction from the aqueous seed fraction. Density centrifugation may be accomplished using decantation centrifuges, including 2-phase and 3-phase decanters, hydrocyclones or disc stack centrifuges. It is also possible to separate the oil body fraction from the aqueous fraction employing size exclusion methods such as membrane ultrafiltration and crossflow microfiltration. The oil bodies may be washed one or more times using preferably water, buffered solutions or other aqueous solutions in order to remove undesirable seed components.

In a further preferred embodiment the somatotropin polypeptide is purified from the seed cells. This is particularly advantageously done by first isolating the oil body fraction as hereinbefore described and subsequently separating the somatotropin polypeptide from the oil body fraction. If a linker comprising a protease cleavage site has been included in the expression cassette, a protease specific for the recognition motif may be added to the oil body preparation. This results in the release of the somatotropin from the oil body. A centrifugation step will result in partitioning of the somatotropin into the aqueous phase. Subsequent purification steps known to the skilled biochemist may be applied to the aqueous fraction in order to obtain a further degree of purity if so desired.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1

Expression of somatotropin in *Brassica napus*

In this example, the expression of carp growth hormone in canola seed is described as well as the purification oil bodies comprising carp growth hormone.

Construction of Oleosin-cGH gene fusion

The fragment of the cGH cDNA (Koren et al., Nucl. Acids Res. 10: 2177-2187) encoding the full length functional protein was modified by site-directed mutagenesis to contain a BamHI and a KpnI site at its 5′ and 3′ ends respectively, and cloned in pUC19 to generate pUCGH. The *Arabidopsis thaliana* 18 kDa oleosin gene containing 800 base pairs of its promoter was previously isolated in our laboratory (US Patent 5,792,922) and engineered to contain sequences encoding a thrombin cleavage site at the 3′ end of the oleosin coding region, followed by a BamHI restriction endonuclease site. This gene was cloned in pUC19 and designated pOthromb (Van Rooijen, 1993, PhD Thesis, University of Calgary). The cGH cDNA was fused to the 3′ end of the oleosin gene using the BamHI site. The fusion construct is shown in Figures 1 and 3.

Figure 1 is a schematic diagram of the oleosin-cGH fusion construct. The oleosin coding sequence, the oleosin promoter sequence and the carp growth hormone cDNA sequence are indicated. A thrombin cleavage site (TCS) is indicated with an arrow. Figure 3 shows the nucleic acid sequence and deduced amino acid sequence of the oleosin-cGH fusion sequence. The deduced amino acid sequence of cGH has been italicized. A thrombin cleavage site has been underlined.

The cassette was subsequently inserted in the multiple cloning site of a plant transformation vector generating the pCGoleoGH800 plasmid.

Plant transformation

The construct pCGOleoGH800 was electroporated into *Agrobacterium tumefaciens* (Dower et al., 1988, Nucl. Acids Res. 16: 6127-6145). A single positive colony was selected and used to transform *B. napus* (cv Westar) cotyledonary explants using the *Agrobacterium*-mediated

transformation method described by Moloney et al., 1989, Plant Cell Reports 8: 238-242 followed by plant regeneration.

Oil body preparation

Seeds were harvested, crushed and homogenized in five volumes of buffer A (100 mM Tris, 500 mM NaCl, 10 mM EDTA, pH 8.0) using a Polytron, and centrifuged at 10,000 x g for 10 minutes. Oil bodies were skimmed from the surface of the supernatant with a metal spatula, re-suspended in buffer and re-centrifuged. Two washes were performed using buffer A, followed by two washes using buffer B (20 mM sodium phosphate, pH 7.3 or PBS as required). Oil bodies were subsequently suspended in buffer B and stored on ice.

Protein Analysis

To extract oil body proteins, oil bodies were boiled in the extraction buffer for 10 minutes. Insoluble material was than removed by centrifugation. Soluble proteins were quantified using the BCA Protein assay (Pierce), and analyzed by 12% SDS-PAGE followed by Western blotting. An anti-cGH rabbit antiserum was used as the primary antibody, and A goat anti-rabbit-IgG [H+L]-AP conjugate (Bio-Rad) was used as the secondary antibody.

The expression of the oleosin-carp growth hormone fusion in three generations of canola seed is shown in Figure 2. Figure 2 shows the SDS-PAGE (Left) and Western Blot (Right) analysis of oil body proteins from seeds of a wild-type (WT) and first (T1), second (T2) and third (T3) generation of plants expressing an oleosin c-GH fusion protein. M; low molecular mass protein markers. A total of 30 μg of protein was loaded into each lane.